ONLINE SUPPLEMENT

ACTIVATION OF NALP3 INFLAMMASOMES TURNS ON PODOCYTE INJURY AND GLOMERULAR SCLEROSIS IN HYPERHOMOCYSTEINEMIA

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Running title: NALP3 Inflammasomes in podocytes

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SUPPLEMENTARY MATERIALS AND METHODS

**Cell culture:** A conditionally immortalized mouse podocyte cell line, kindly provided by Dr. Klotman PE (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA), was cultured on collagen I-coated flasks or plates in RPMI 1640 medium supplemented with recombinant mouse interferon–γ at 33°C. After differentiation at 37°C for 10-14 days without interferon–γ, podocytes were used for the proposed experiments. L-Hcys (a pathogenic form of Hcys) was used and its concentration and incubation time in cell culture dishes were chosen based on our previous studies \(^1\) and some preliminary experiments.

**ASC siRNA transfection:** ASC siRNA was purchased from Qiagen (Valencia, CA, USA), which was confirmed to be effective in silencing the ASC gene in different cells by the company. The scrambled RNA (Qiagen, Valencia, CA, USA) was also confirmed as non-silencing double-strand RNA and was used as a control. Podocytes were serum-starved for 12 h and then transfected with ASC siRNA or scrambled siRNA using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA). After 18 h of incubation at 37°C, the medium was changed and L-Hcys (40 µmol/L) added into the medium for indicated time spans in different protocols.

**Real-time reverse transcription polymerase chain reaction (RT-PCR):** Total RNA from cultured podocytes or isolated mouse glomeruli was extracted using TRizol reagent (Invitrogen, Carlsbad, CA. USA) according to the protocol described by the manufacturer. The primers used in this study were synthesized by Operon (Huntsville, AL, USA) and the sequences were: for NALP3 sense TACGGCCGTCTACGTCTTCT, antisense CGCAGATCACCCTCTCAAAC; for ASC sense ACAGAAGTGGACGGAGTGCAC, antisense CTCCAGGTCCATCACCAAGT; for caspase-1 sense CACAGCTCTGGAGATGTTGA, antisense TCTTTCAAGCTTGGCACTTT; for nephrin sense CCCGGACACCTGTATGACGAG, antisense CCGCCACCTTGTCGTTGATT; for desmin sense CATACCCCTAGAGATT, antisense GGCCATCTTCACATGGACG; and for β-actin sense TCGCTGCGCTGGTCGCT, antisense GGCCTCGTCACCCACATAGA.

**Caspase-1 activation assay:** Caspase-1 activity was detected using a commercially available kit (Biovision, Mountain View, CA, USA), which was used to represent activation of NALP3 inflammasomes. The data was expressed as the fold changes compared with control cells.

**Indirect immuno-fluorescent staining and confocal microscopy:** For colocalization of inflammasome molecules in podocytes, cultured cells were fixed in 4% PFA for 15 minutes. After being rinsed with phosphate-buffer saline (PBS), the cells were incubated overnight at 4°C with goat anti-NALP3 (1:200, Abcam, Cambridge, MA, USA) and rabbit anti-ASC (1:50, Enzo, Plymouth Meeting, PA), or goat anti-NALP3 (1:200) and anti-caspase-1 (1:100,
Abcam, Cambridge, MA, USA). To colocalize inflammasome molecules and podocyte markers in the mouse kidney, double-immunofluorescent staining was performed using frozen tissue slides. After fixation, the slides were incubated overnight at 4°C with goat anti-NALP3 (1:200) and rabbit anti-ASC (1:50), or goat anti-NALP3 (1:200) and anti-caspase-1 (1:100). In additional experiments, tissue slides were probed with anti-NALP3 or caspase-1 antibody together with anti-podocin antibody (1:400, Sigma, St. Louis, MO, USA) to show the localization of inflammasome molecules in podocytes. After washing, these slides probed with primary antibodies were incubated with Alexa-488- or Alexa-555-labeled secondary antibodies for 1 h at room temperature. After being mounted and the colocalization of NALP3 with ASC or caspase-1 analyzed by the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The data was expressed as Pearson correlation coefficient (PCC) as we described previously 2.

Size-exclusion chromatography (SEC): Podocytes or isolated glomeruli were homogenized with the following protein extraction buffer: 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L Na EDTA, 1 mmol/L Na EGTA, and 1× protease inhibitor cocktail set I (Calbiochem, Gibbstown, NJ, USA). Samples were then centrifuged at 18,000 g for 10 min at 4°C and run on a Sepharose 6 size-exclusion chromatography column with the following buffer: 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% octylglucoside, and 1× protease inhibitor cocktail. Fractions (200 μl) were collected starting at the void volume time. 5× sample buffer was added directly to the fractions, which were then heated at 95°C for 5 min and resolved in SDS-polyacrylamide gel electrophoresis gels followed by Western blot analysis 3. Protein standards were run on a column under identical conditions and absorbance at 280 nm was used to analyze the fractions.

Western blot analysis: Protein from podocyte lysate or mouse kidney tissues was run on an SDS-PAGE gel, transferred on to PVDF membrane and blocked. Then, the membrane was probed with primary antibodies against NALP3, ASC, or caspase-1 (1:500 dilution) overnight at 4°C followed by incubation with horseradish peroxidase-labeled immunoglobulin G. The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat film. β-actin was reprobed to serve as a loading control. The intensity of the bands was quantified by densitometry.

Direct fluorescent staining of F-actin: To determine the role of NALP3 inflammasome activation in Hcys-induced cytoskeleton changes, podocytes were cultured in 8-well chambers. After pretreatment with vehicle, caspase-1 inhibitor (Z-WEHD-FMK) or transfected with ASC siRNA or scrambled siRNA, the cells were treated with L-Hcys (40 μmol/L) or puromycin aminonucleoside (PAN, 100 μg/mL, Sigma, St. Louis, MO, USA) for 24 h. After washing with PBS, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and blocked with 3% bovine serum albumin. F-actin was stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA,
USA) for 15 min at room temperature. After mounting, the slides were examined by a confocal laser scanning microscope. Cells with distinct F-actin fibers were counted as we described previously. Scoring was obtained from 100 podocytes on each slide in different groups.

**ELISA for vascular endothelial growth factor A (VEGF-A) and IL-1β in podocytes and glomeruli:** After transfection with ASC siRNA, scrambled siRNA, or pretreatment with Z-WEHD-FMK and its vehicle, podocytes were incubated with L-Hcys (40 µmol/L) for 24 h. A specific podocyte injury compound, puromycin aminonucleoside (PAN, 100 µg/ml) was used to treat cells for 24 h to serve as a positive control. The supernatant was collected for ELISA assay of VEGF-A using a commercially available kit (R&D system, Minneapolis, MN). In additional experiments, IL-1β in the cell supernatant and mouse glomeruli were measured by a mouse IL-1β ELISA kit from Bender Medsystems (San Diego, CA, USA) according to the protocol described by the manufacturer.

**Transmission electron microscopy (TEM):** For TEM observation of ultrastructural changes in podocytes, the kidneys were perfused with a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer. After fixation and dehydration with ethanol, the samples were embedded in Durcupan resin for ultra-thin sectioning and TEM examination by VCU electron microscopy core facility.

Isolation of mouse glomeruli, morphological examination, mean arterial pressure, plasma homocysteine, urinary albumin and creatinine measurements were performed as we described previously.

**Statistical analysis:** All of the values are expressed as mean ± SEM. Significant differences among multiple groups were examined using ANOVA followed by a Student-Newman-Keuls post hoc test. \( \chi^2 \) test was used for testing the significance of ratio and percentage data. \( P<0.05 \) was considered statistically significant.
SUPPLEMENTARY RESULTS

Effects of ASC gene silencing and caspase-1 inhibition on functional changes induced by L-Hcys. Using rhodamine-phalloidin to stain F-actin, podocytes treated with L-Hcys were found to have reduced and reorganized F-actin fibers, which was normally distributed along the longitudinal axis within the podocytes. In podocytes treated with L-Hcys, the remaining F-actin fibers were found mainly aggregated around the periphery of the cells, which was similar to the changes induced by a podocyte-selective toxic compound. After ASC gene silencing or inhibition of caspase-1 activity, the amount and distribution pattern of F-actin in podocytes were restored to normal (Figure S1). These changes in F-actin staining were summarized in Figure S1.

Efficiency of in vivo local transfection of ASC shRNA into the kidney. As shown in Figure S3A, the expression efficiency of the co-transfected luciferase gene was monitored daily using an in vivo molecular imaging system to insure an efficient introduction of target gene into the kidney cells. Even on the 4th day after the transfection, the gene expression could be detected. This expression continued for 4 weeks. As shown in a hemi-dissected kidney, almost all of the cortical regions exhibited efficient gene transfection, as shown in green fluorescence compared with the control kidney (Figure S3B). RT-PCR analysis demonstrated that FF diet treatment for 4 weeks significantly increased the ASC mRNA expression, which was substantially blocked in ASC shRNA-transfected glomeruli both on the normal diet or FF diet, indicating the successful silencing of ASC gene in mouse glomeruli (Figure S3C).
SUPPLEMENTARY REFERENCES


Table S1: Body weight, plasma Hcys concentration, mean arterial pressure and heart rate in C57BL/6J WT mice transfected with or without ASC shRNA and fed a ND or FF diet (n= 4-5).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Scra ND</th>
<th>ASCsh ND</th>
<th>Scra FF</th>
<th>ASCsh FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (gm)</td>
<td>27.7 ± 1.3</td>
<td>28.4 ± 1.5</td>
<td>27.8 ± 1.2</td>
<td>25.2 ± 0.6</td>
</tr>
<tr>
<td>Hcys Conc. (µM)</td>
<td>6.1 ± 1.2</td>
<td>6.8 ± 0.3</td>
<td>17.5 ± 1.1*</td>
<td>20.1 ± 2.1*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>102.4 ± 3.4</td>
<td>107.5 ± 1.4</td>
<td>105.7 ± 2.0</td>
<td>105.4 ± 0.8</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>563.2 ± 11.4</td>
<td>580.8 ± 8.0</td>
<td>581.4 ± 3.0</td>
<td>558.2 ± 13.0</td>
</tr>
</tbody>
</table>

ND: Normal diet, FF: Folate-free diet, MAP: Mean arterial pressure, Hcys: Homocysteine, ASCsh: ASC shRNA, Scra: Scrambled shRNA-transfected. The data was expressed as mean ± SEM. *p<0.05 as compared with Scra ND.
Figure S1. A. Representative microscopic images showing F-actin staining in podocytes using rhodamine-phalloidin staining (magnification, ×400). B. Summarized data showing the rate of podocytes retaining distinct longitudinal stress fibers. Scoring was from 100 podocytes on each slide in different groups (n=6 batches of podocytes). Ctrl: control; Veh: vehicle; Scra: scrambled siRNA; Casp-1: Caspase-1; WEHD: Z-WEHD-FMK. * P<0.05 vs. control; # P<0.05 vs. Hcys.
Figure S2. Formation and activation of NALP3 inflammasomes in glomeruli of hHcys mice. A. Western blot analysis of protein fractions obtained from glomeruli of normal diet and FF diet fed mice probed with anti-NALP3 and ASC antibodies. B. Summarized data showing the band intensities measured from the inflammasome complex fractions (fractions 7-21) of NALP3 and ASC (n=3). C. Total and cleaved caspase-1 expression in mouse glomeruli after 4 weeks of FF or normal diet treatment. D. Summarized data showing the quantification of cleaved caspase-1 expression, which was normalized to β-actin (n=7). Casp-1: Caspase-1, N diet: Normal diet, * P<0.05 vs. ND.
**Figure S3.** *In vivo* and *in vitro* determination of gene transfection efficiency in the kidney. 
A. Daily imaging confirmation of gene transfection in the kidney by an *in vivo* molecular imaging system. B. Localization of transfected gene expression in the hemi-dissected kidney on day 14 after gene delivery. C. Real-time RT-PCR detection of ASC mRNA after ASC shRNA delivery at 4 weeks after gene delivery (n=4). * P<0.05, vs. scrambled shRNA-transfected mice on the N diet; # P<0.05, vs. scrambled shRNA-transfected mice on the FF diet.
Figure S4. A. Colocalization of NALP3 (green) with podocin (red) and caspase-1 (green) with podocin (red) in mouse glomeruli. B. Summarized data showing the fold changes in PCC for the colocalization of podocin with NALP3 or caspase-1. C. Real-time RT-PCR analysis showing changes in the expression of nephrin and desmin in the glomeruli from different groups of mice (n=4 per group). D. Immunofluorescent staining of nephrin and desmin in the glomeruli from 6 groups of mice (n=6 per group). N Diet: Normal Diet; Casp-1: Caspase-1; Scra: Srambled shRNA-transfected; ASC sh: ASC shRNA-transfected. *P<0.05, vs. scrambled shRNA-transfected mice on the N diet (normal diet); # P<0.05, vs. scrambled shRNA-transfected mice on the FF diet. (n=6).